TITLE OF THE INVENTION

Glanders/Meliodosis Vaccines

This application claims the benefit of priority under 35 U.S.C. 119(e) from U.S. Application Serial No. 60/396,257 filed on July 15, 2002, still pending.

INTRODUCTION

Burkholderia mallei, the etiologic agent of glanders disease is a gram-negative, oxidase positive, nonmotile bacillus that is an obligate animal pathogen (DeShazer, D and D. M. Wang, 2002, New Insights into an Old Disease. In L. Lindler et al. eds., Biological Weapons Defense: Principes and Mechanisms for Infectious Diseases Counter-Bioterrorism. The Humana Press Inc.). The natural hosts of *B. mallei* are horses, donkeys, and mules (solipeds) while humans are considered an incidental host (DeShazer and Wang, 2002, supra). Until the early 20th century, and the development of motorized transportation, glanders disease was prevalent worldwide (DeShazer and Wang, 2002, supra). With the requirement of quaratinement of imported animals, no naturally occurring cases of glanders have been reported in the United States since 1934 (DeShazer and Wang, 2002, supra). Human glanders is uncommon, and occasionally occurs in individuals (veterinarians, slaughter house workers, and laboratory scientists) whose occupation puts them at risk (Steele, J. H., 1979, In: Steele JH, ed. CRC Handbook Series in Zoonoses. Boca Raton, FL: CRC Press, 339-362). In solipeds, two distinctive forms of glanders may arise, chronic (common in horses) and acute (observed in mules and donkeys). Symptoms of acute glanders include weight loss, difficulty breathing, and elevated temperature. In contrast, horses with chronic glanders may exhibit pulmonary, cutaneous (farcy), and respiratory symptoms. Human acute glanders is characterized by fever, fatigue, and

inflammation and nodule formation on the face and peripheral limbs (DeShazer and Wang, 2002, supra). Symptoms of chronic glanders in humans consist of swollen lymph nodes, ulcerating nodules in the alimentary and respiratory tracts, and numerous subcutaneous abscesses (DeShazer and Wang, 2002, supra).

Burkholderia pseudomallei, the causative agent of melioidosis, inflicts high incidences of human pneumonia and deadly bacteremia in endemic areas including Southeast Asia and northern Australia (Woods, D. E. et al., 1999, Microbes Infect. 2, 157-162; Dance, D.A., 2002, Melioidosis, Curr. Opin. Infect. Dis. 2, 127-132). Interestingly, recent studies have successfully isolated *B. pseudomallei* from both the environment and humans in areas of Europe, Africa, the Middle East, and central and South America (Woods,DE et al., 1999, supra). *B. pseudomallei* is a gram-negative soil saprophyte and is a common inhabitant of surface waters and soil (Ulett, GC et al., 2001, Microbes Infect. 3, 621-631). Disease in humans normally occurs in individuals who are frequently exposed to contaminated surface water and soil, in particular rice farmers in Thailand and the Aboriginal people in Australia (Ulett et al., 2001, supra). Several underlying host conditions including diabetes, renal complications, and alcoholism are additional risk factors for contracting *B. pseudomallei* (Ulett et al., 2001, supra). Symptoms of melioidosis are discrete and may include acute or chronic pneumonia, acute septicemia and even latent infections that can persist for several years (Ulett et al., 2001, supra).

Aerosol exposure to *B. mallei* and *B. pseudomallei* results in sinus cavity colonization, followed by dissemination into the blood stream and peripheral organs in animal models of infection. Because biofilm maturation is

probably important for sinus colonization, mutants impaired in biofilm progenesis may be hindered in their aerosol pathogenicity and may give insight into the unique aspects of these pneumonic diseases.

The bacterial quorum sensing cascade has been shown to be critical for regulating many cell-density dependant processes, including biofilm maturation. The quorum gene systems found in numerous gram-negative bacteria are sophisticated cell-cell signaling pathways that allow a microorganism to detect and respond at the transcriptional level to fluctuating environmental conditions (Fuqua, C. et al., 2001, Annu. Rev. Genet. 35, 439-468). Briefly, quorum systems operate using synthase enzymes (AHS) that produce small signaling molecules termed *N*-acyl-homoserine lactones (AHL), which bind to transcriptional regulatory proteins (LuxR) and activate or repress gene expression. Often, bacterial species possess multiple quorum networks and the interaction between the diffrent systems adds complexity and flexibility to gene expression.

This ability to transduce intracellular signals, termed quorum-sensing, involves the synthesis and accumulation of AHLs (Fuqua, C. et al., 2001, supra). AHLs are secreted into the extracellular medium and diffuse back into the cell when a high concentration has been reached. AHL biosynthesis is enzymatically mediated by the LuxI family of proteins and a single LuxI may produce multiple AHLs with varying side chain lengths (Fuqua, C. et al., 2001, supra). LuxR proteins respond to AHLs in a concentration dependent manner through the binding of the signal molecule. This protein interaction induces conformational changes and multimerization of the enzyme, which in turn induces or represses target gene expression (Fuqua, C. et al., 2001, supra). In animal and plant pathogens, coordinated gene expression, in particular alleles encoding proteins needed

for virulence, allows microorganisms to elicit an overwhelming attack before host cells can mount an effective defense (Fuqua, C. et al., 2001, supra).

In *Pseudomonas aeruginosa*, two thoroughly characterized quorum networks have been analyzed at the genetic and biochemical levels and consist of the *lasIR* and *rhlIR* systems (Fuqua, C. et al., 2001, supra). Collectively, these quorum networks direct the synthesis of *N*-3-oxodo-decanoyl homoserine lactone (LasI) and *N*-butyryl-homoserine lactone (RhlI) and encode the transcriptional regulators for elastase (LasR) and rhamnolipid (RhlR) biosynthesis (Fuqua, C. et al., 2001, supra). Disruption of the *P. aeruginosa lasIR* and *rhlIR* systems significantly reduces the virulence in multiple animal models including acute and chronic lung infections in neonatal mice and adult rats (Smith, R. S. et al., 2002, J. Bacteriol. 184, 1132-1139). Additionally, several investigations have demonstrated that *N*-3-oxodo-decanoyl homoserine lactone accumulation *in vitro* and *in vivo* promotes the induction of numerous inflammatory mediators that result in tissue destruction and subsequent dissemination of *P. aeruginosa* to peripheral organs (Smith, R.S. et al., 2002, supra).

Lewenza et al. and Conway et al. recently identified functional quorum-sensing networks in *Burkholderia cepacia* and *Burkholderia vietnamiensis* (Lewenza, S. et al., 1999, J. Bacteriol. 181, 748-756; Lewenza, S. and P. A. Sokol 2001, J. Bacteriol. 183, 212-218; Conway, B. and E.P. Greenberg, 2002, J. Bacteriol. 184, 1187-1191). The *B. cepacia* quorum system is comprised of the *cepIR* loci. CepI directs the biosynthesis of *N*-octanoyl-homoserine lactone (C_8-HSL) and *N*-hexanoyl-homoserine lactone (C_6-HSL) (Lewenza et al.,

1999, supra). Mutational analysis of the *cepIR* system demonstrated that CepR negatively regulated ornibactin synthesis and positively induced protease and C_8-HSL biosynthesis (Lewenza and Sokol, 2001, supra). These findings indicate that quorum sensing in *B. cepacia* positively and negatively regulates potential virulence factors using a cell density mechanism.

In an effort to identify quorum alleles encoded by both *B. mallei* and *B. pseudomallei*, an *in silico* approach was pursued that used the LasIR, RhlIR, and the CepIR amino acid sequences to search the *B. pseudomallei* K96243 (http://www.sanger.ac.uk/DataSearch/) and the *B. mallei* ATCC 23344 (http://tigrblast.tigr.org/ufmg) genomes for quorum sensing homologues.

BLAST search revealed that the *B. pseudomallei* genome encodes three AHS genes (*bpmI1, bpmI2, and bpmI3*) and five transcriptional regulators (*bpmR1, bpmR2, bpmR3, bpmR4*, and *bpmR5*) belonging to the LuxR family of proteins. In contrast, B. mallei contains two AHS genes (*bmaI1 and bmaI2*) and four LuxR homologues (*bmaR1, bmaR3, bmaR4, bmaR5*). The relative genetic organization of these complex quorum sensing operons are shown in Figure 1. Interestingly, *B. mallei* is lacking the entire *bpmIR2* locus and the flanking open reading frames (orf). The genes encoded within these operons show an interesting arrangement relative to other characterized quorum systems in gram-negative bacteria. Usually, the AHS and LuxR genes are arranged in an uninterrupted tail-to-tail orientation. None of the identified loci display this arrangement. Further, there are LuxR genes (*bmaR4, bmaR5, bpmR4*, and *bpmR5*) encoded by both *B. mallei* and *B. pseudomallei* that are orphaned for a

cognate AHS. Typically, both genes are found together and interact with each other. Based upon the *in silico* recovered quorum alleles, we selected oligonucleotide primers for the amplification of an internal fragment of approximately 400 bp in each gene (Table 2). We cloned the internal fragments following amplification using a topoisomerase mediated method. The internal fragments were subcloned into a suicide plasmid bearing a gentamycin resistance marker for mobilization via *E. coli* into *B. mallei and B. pseudomallei* (Sambrook, J. et al., 1989, Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Simon, R. et al., 1983, Bio/technology 1, 784-791; DeShazer, D. et al., 2001, Microb. Pathog. 30, 253-269). A single crossover event between the internal gene fragment and the bacterial chromosome would generate a merodiploid, disrupt the targeted gene and give the recipient a gentamycin resistant phenotype. Merodiploids were constructed in each of the *B. mallei* and *B. pseudomallei* quorum alleles and the resulting strains were phenotypically characterized in vivo (see Table 1 for bacteria and strains used in this study). The *B. pseudomallei* allele for each gene to be disrupted was used in the construction of the insertional mutagenesis cassette. Utilization of this strategy allowed the generation of mutants with the smallest number of mutagenesis cassette clones.

Interestingly, both the GB8::*bpmI1* and GB8::*bpmI3* mutants were avirulent even though they both produced a capsule. To date the only definitive virulence factor associated with pathogenicity of *B. mallei* is extracelllar capsule (DeShazer, D. et al., 2001, supra). All of the *B. mallei* quorum sensing mutants tested in this study produce

capsule even those with reduced virulence. This is of significant importance indicating that this study has identified novel and previously unknown regulators of virulence and virulence gene expression.

The reduction in the ability of all the *B. mallei* quorum sensing mutans, in particular, GB8::*bpmI3* and GB8::*bpmI1*, to colonize the spleen, liver, and lungs of aerosolized BALB/c mice indicated that quorum sensing plays a pivotal role in the pathogenicity of *B. mallei* in vivo. Exposure of animals to GB8::*bpmI3* mutants prior to challenge protected approximately 40% of animals over a 21 day period while all unvaccinated animals perished within 3 days. There are no published reports showing the efficacy of a subunit or live attenuated strain of *B. mallei* providing any protection against an aerosol challenge with wild-type.

This was an unexpected result. Given that the capsule mutants were avirulent, we expected that they would protect. However, they do not. In retrospect, it seems that the quorum mutants retain enough vigor to activate the host immune system while the capsule mutants were simply cleared. The majority of clinical data previously reported suggests that the capsule and LPS would make a good vaccine, but all preparations of components to date have failed to yield sterile immunity following an aerosol challenge.

SUMMARY OF THE INVENTION

We have cloned and characterized 8 previously unknown quorum genes from *B. pseudomallei* DD503, and using this information were able to clone and characterize 6 new quorum genes from *B. mallei* ATCC 23344. We have shown that disruption of genes with the *bmaIR* locus results in avirulent strains of *B. mallei* and that some of these

strains can effectively be used as a vaccine against glanders disease.

Therefore, it is one object of the present invention to provide a DNA fragment encoding each of the *B. mallei* AHS and AHS transcriptional regulators (LuxR) quorum genes, *bmaI1* (612 bp, SEQ ID NO:1), *bmaI3* (609 bp, SEQ ID NO:2), *bmaR1* (720 bp, SEQ ID NO:3) *bmaR3* (609 bp, SEQ ID NO:4), *bmaR4* (660 bp, SEQ ID NO:5), and *bmaR5*(726 bp, SEQ ID NO:6).

It is another object of the present invention to provide a DNA fragment encoding each of the *B. pseudomallei* quorum genes, *bpmI1* (612 bp, SEQ ID NO:7), *bpmI2* (621 bp, SEQ ID NO:8), *bpmI3* (609 bp, SEQ ID NO:9), *bpmR1* (720 bp, SEQ ID NO:10), *bpmIR2* (711 bp, SEQ ID NO:11), *bpmR3* (693 bp, SEQ ID NO:12), *bpmR4* (885 bp, SEQ ID NO:13), *bpmR5* (726 bp, SEQ ID NO:14).

It is another object of the present invention to provide the DNA fragments mentioned above in a recombinant vector. When the vector is an expression vector, the *Burkholderia* proteins encoded by the DNA fragments are produced. The DNA fragments are useful as a diagnostic agent, an agent for preparation of the protein which it encodes, and as a therapeutic agent. Specifically, the *bpmIR2* genes, which *B. mallei* lacks, will provide an ideal diagnostic target to distinguish *B. pseudomallei* from *B. mallei*.

It is another object of the invention to provide an amino acid sequence encoded by the DNA sequences above.

It is a further object of the present invention to provide a host cell transformed with the above-described recombinant DNA construct.

It is another object of the present invention to provide a method for producing the above-mentioned AHSs and AHS transcriptional regulators encoded by the DNA fragments above, the method comprising culturing a host cell under conditions such that the above-described DNA fragment is expressed and the encoded protein is thereby produced, and isolating the protein for use as a reagent, for example for screening of drugs and inhibitors of AHS, for drugs and inhibitors that compete or inhibit the binding of the AHS to the LuxR homologues, for drugs and inhibitors of the LuxR transcriptional regulators, or for inhibiting the AHS quorum sensing operon.

It is a further object of the present invention to provide an antibody to the above-described recombinant proteins.

It is yet another object of the present invention to provide a method for detecting AHS in a sample comprising:

(i) contacting a sample with antibodies which recognize AHS; and

(ii) detecting the presence or absence of a complex formed between AHS and antibodies specific therefor.

It is yet another object of the present invention to provide a method for detecting AHS transcriptional regulator in a sample comprising:

(i) contacting a sample with antibodies which recognize AHS transcriptional regulator; and

(ii) detecting the presence or absence of a complex formed between AHS transcriptional regulator and antibodies specific therefor.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody against AHS and ancillary reagents suitable for use in detecting the presence of AHS in a sample, e.g. tissue or serum from,

mammals including humans, animals, birds, fish, plants and fungi, air, soil, or water.

It is yet another object of the present invention to provide a method for the detection of AHS, or LuxR transcription regulators in a sample using the polymerase chain reaction.

It is a further object of the present invention to provide a diagnostic kit comprising primers or oligonucleotides specific for AHS RNA or cDNA suitable for hybridization to AHS RNA or cDNA and/or amplification of AHS sequences and ancillary reagents suitable for use in detecting AHS RNA/cDNA in a sample.

It is yet another object of the present invention to provide a method for the detection of AHS in a sample which comprises assaying for the presence or absence of AHS RNA or cDNA in a sample by hybridization assays.

It is yet another object of the present invention to provide a method for reducing *Burkholderia* virulence by inhibiting the expression of one or more AHS in said cell. The inhibition can be at the DNA level by introducing mutations into the gene encoding one or more AHS, by inhibiting transcription of the gene, by inhibiting translation of the RNA encoding one or more AHS, or by inhibiting the function of one or more AHS.

It is yet another object of the present invention to provide a method for reducing *Burkholderia* virulence by inhibiting the expression of one or more LuxR transcriptional regulator in said cell. The inhibition can be at the DNA level by introducing mutations into one or more gene encoding one or more transcriptional regulator, by inhibiting transcription of the gene, by inhibiting translation of the RNA encoding a trancriptional regulator,

or by inhibiting the function of one or more transcriptional regulator.

It is a further object of the present invention to provide *Burkholderia* strains containing one or more alteration in one or more AHS or LuxR quorum gene sequence. Such alteration can be insertions, deletions, or substitutions.

It is another object of the present invention to provide *B. mallei* or *B. pseudomallei* strains containing a non-revertable mutation within any of the AHS genes and/or LuxR gene for use in a vaccine composition.

It is yet another object of the present invention to provide a method and composition to elicit *Burkholderia* specific immune response in an individual comprising administering to the individual GB8::*bpmI3* or a strain with a non-revertable mutation in *bmaI3* in an amount sufficient to induce such a response.

It is another object of the present invention to provide a method for making an avirulent strain of *B. mallei* or *B. pseudomallei*, comprising disrupting one or more AHS gene and/or one or more LuxR gene.

It is further an object of the invention to provide an immunological composition for the protection of subjects against aerosolized glanders infection comprising *B. mallei* containing one or more disruption in one or more AHS gene and/or one or more LuxR gene allele, wherein said disruption is due to an insertion or deletion or substitution in the AHL synthase allele.

It is still another object of the present invention to provide a method for identifying downstream components or interacting proteins important for the virulence of *B. mallei* or *B. pseudomallei* activated by AHS or LuxR genes, by

identifying genes expressed in the wild type *Burkholderia* but not in a mutant avirulent strain having a mutation in one or more AHS genes and/or one or more LuxR genes.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings.

Figure 1. Structural organization of the *B. mallei* ATCC 23344 quorum sensing network. The ASH genes are represented as *bmaI1* and *bmaI3* and the *luxR* homologues are labeled as *bmaR1*, *bmaR3*, *bmaR4*, and *bmaR5*. ORF depicts a potential open reading frame. The surrounding genes are putative orfs identified by performing tblastn searches (http://www.ncbi.nlm.nih.gov/BLAST/).

Figure 2. Structural organization of the *B. pseudomallei* K96234 quorum sensing network. The ASH genes are represented as *bpmI1*, *bpmI2* and *bpmI3* and the *luxR* homologues are labeled as *bmaR1*, *bmaR2*, *bmaR3*, *bmaR4*, and *bmaR5*. ORF depicts a potential open reading frame. The surrounding genes are putative ORFs identified by performing tblastn searches (http://www.ncbi.nlm.nih.gov/BLAST/).

Figure 3A, 3B, 3C. The organ loads of female BALB/c mice aerosolized with *B. mallei* ATCC 23344 quorum sensing mutants. (A) represents the the number of viable organisms within the lungs, (B) depicts CFUs recovered from the spleen, and (C) demonstrates the organ loads in the liver. Animals were challenged with approximately 10^5 CFUs of wild-type *B. mallei* ATCC 23344 and each quorum sensing mutant. Organs were extracted at days 1-5 and at day 30 post challenge. GB15 represents wild-type *B. mallei* ATCC 23344.

Figure 4. Time to death of BALB/c mice infected with wild type *B. mallei* ATCC 23344 and each quorum sensing mutant. Female BALB/c mice were aerosolized with approximately 10^5 CFUs of wild type *B. mallei* ATCC 23344 and each derivative quorum sensing mutant. Animal death was followed over a 29 day interval. GB15 represents wild type *B. mallei*.

Figure 5. Time to death of BALB/c mice infected with wild type *B. pseudomallei* DD503 and each quorum sensing mutants. Female BALB/c mice were aerosolized with approximately 10^5 CFUs of wild type *B. pseudomallei* DD503 and each derivative quorum sensing mutant. Animal death was followed over a 29 day interval. DD503 represents wild type *B. pseudomallei*.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to DNA fragments which encode *B. mallei* AHS genes, *bmaI1* (SEQ ID NO:1), and *bmaI3* (SEQ ID NO:2) which are involved in the synthesis of N-acyl-homoserine lactones (AHL) and LuxR genes or DNA fragments which encode transcriptional regulatory proteins *bmaR1* (SEQ ID NO:3), *bmaR3* (SEQ ID NO:4), *bmaR4* (SEQ ID NO:5), and *bmaR5* (SEQ ID NO:6) which bind signals produced by AHS and activate or repress gene expression.

This invention further relates to DNA fragments encoding *B. pseudomallei* AHS genes, *bpmI1* (SEQ ID NO:7), *bpmI2* (SEQ ID NO:8), and *bpmI3* (SEQ ID NO:9) and LuxR genes which encode transcriptional regulatory proteins *bpmR1* (SEQ ID NO:10), *bpmR2* (SEQ ID NO:11), *bpmR3* (SEQ ID NO:12), *bpmR4* (SEQ ID NO:13), and *bpmR5* (SEQ ID NO:14).

In addition, this invention relates to the amino acid sequence of *B. mallei* AHS, BmaI1 (SEQ ID NO:15), and BmaI3

(SEQ ID NO:16) which are involved in the synthesis of N-acyl-homoserine lactones (AHL) and transcriptional regulatory response proteins BmaR1 (SEQ ID NO:17), BmaR3 (SEQ ID NO:18), BmaR4 (SEQ ID NO:19), and BmaR5 (SEQ ID NO:20). This invention further relates to the amino acid sequence of *B. pseudomallei* AHS, BpmI1 (SEQ ID NO:21), BpmI2 (SEQ ID NO:22), and BpmI3 (SEQ ID NO:23) and transcriptional regulatory response proteins BpmR1 (SEQ ID NO:24), BpmR2 (SEQ ID NO:25), BpmR3 (SEQ ID NO:26), BpmR4 (SEQ ID NO:27), and BpmR5 (SEQ ID NO:28).

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from (a) a nucleotide sequence comprising a sequence encoding a full length AHS polypeptide from *B. mallei* or *B. pseudomallei* having the sequence specified in SEQ ID NO:1-14, (b) a nucleotide sequence which encodes the complete amino acid sequence in SEQ ID NO:15-28.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the protein or fragments thereof. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in a human mRNA to those preferred by a bacterial host such as *E.coli*).

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may

be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By ``isolated'' nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequence of SEQ ID NO:1-14 or at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence of the gene minus 1.

Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from intergers between 1- and the entire length of an entire nucleotide sequence minus 1. Preferred sizes include 20-50

nucleotides, 50-300 nucleotides useful as primers and probes. Regions from which typical sequences may be derived include but are not limited to, for example, regions encoding specific domains within said sequence, such as the region comprising the active domain of the enzyme, or the domain which binds the transcriptional regulatory protein.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a polynucleotide sequence of the present invention described above, or a specified fragment thereof. By ``stringent hybridization conditions'' is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at about 65°C.

The sequences encoding the polypeptides of the present invention or portions thereof may be fused to other sequences which provide additional functions known in the art such as a marker sequence, or a sequence encoding a peptide which facilitates purification of the fused polypeptide, peptides having antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-tranlational modifications, or amino acid sequences which target the fusion protein to a desired location, e.g. a heterologous leader sequence.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the polypeptides. Variant may occur naturally, such as a natural allelic variant. By an ``allelic variant'' is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occurring

variants may be produced by known mutagenesis techniques. Such variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid substitutions, deletions, or additions.

Nucleic acid molecules with at least 90-99% identity to any nucleic acid shown in any of SEQ ID NO:1-14 is another aspect of the present invention. These nucleic acids are included irrespective of whether they encode a polypeptide having AHS activity or AHS transcriptional regulator protein activity. By ``a polypeptide having AHS activity'' is intended polypeptides exhibiting activity similar, but not identical, to an activity of the AHS of the invention, as measured in the assays described below. By ``a polypeptide having AHS transcriptional regulator activity'' is intended polypeptides exhibiting activity similar, but not identical, to an activity of the transcriptional regulator of the invention, as measured in the assays described below. The biological acitivity or function of the polypeptides of the present invention are expected to be similar or identical to polypeptides from other organisms that share a high degree of structural identity/similarity. There are different strains of *Burkholderia*. The AHS and AHS transcriptional regulator genes of these different strains have not been sequenced. It would be expected that these proteins would have homology among different strains and that vaccination against one *Burkholderia* strain might afford cross protection to other *Burkholderia* strains.

In another embodiment, the present invention provides allelic variants wherein the gene has been altered for the purpose of reducing or eliminating activity of the gene

product, i.e. the AHS or the AHS transcriptional regulator. Such negative allelic variants can be produced by the methods described by Moore et al. (Moore, R. A. et al., 1999, Antimicrob. Agents Chemother. Mar 43, 465-470). It is conceivable that a single derivative of *B.mallei* containing multiple deletions, for example in the AHS genes, *bmaI3* and *bmaI1*, and the LuxR gene *bmaR5*, will display a combined phenotype that results in an extremely attenuated, or an avirulent strain of *B. mallei*. Such an a strain can be used in a vaccine composition as described below.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. Among the vectors preferred for use include pCR2.1-TOPO, pGSV3, to name a few. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. *et al*. (Eds), Wiley &

Sons, Inc. All documents cited herein *supra* and *infra* are hereby incorporated in their entirety by referece thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of the encoded protein, such as glutathione S-transferase, or a series of histidine residues also known as a histidine tag. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces*

carlsbergensis, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as HEK293 cells , and NIH 3T3 cells, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate. Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to a AHS protein having an amino acid sequence corresponding SEQ ID NO:15, 16, 21, 22, and 23 or any allelic variation thereof or biologically active or biologically inactive derivative thereof. The present invention further relates to AHS transcriptional regulator protein having an amino acid sequence corresponding to SEQ ID NO:17-20, 24-28 or any

allelic variation thereof or biologically active or biologically inactive derivative thereof.

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A ``biologically active derivative thereof'' is a AHS or a AHS transcriptional regulator that is modified by amino acid deletion, addition, substitution, or truncation, or that has been chemically derivatized, but that nonetheless functions in the same manner as any protein of SEQ ID NO:15-28. For example, it is known that substitutions of aliphatic amino acids such as alanine, valine, and isoleucine with other aliphatic amino acids can often be made without altering the structure or function of a protein. Similarly, substitution of aspartic acid for glutamic acid, in regions other than the active site of an enzyme, are likely to have no appreciable affect on protein structure or function. The term ``fragment'' is meant to refer to any polypeptide subset. Fragments can be prepared by subjecting *Burkholderia* proteins to the action of any one of a number of commonly available proteases, such as trypsin, chymotrypsin or pepsin, or to chemical cleavage agents, such as cyanogen bromide. The term ``variant'' is meant to refer to a molecule substantially similar in structure and function to either the entire AHS or AHS transcriptional regulator or to a fragment thereof. A protein or peptide is said to be `substantially similar' if both molecules have substantially similar amino acid

sequences, preferably greater than about 80% sequence identity, or if the three-dimensional backbone structures of the molecules are superimposable, regardless of the level of identity between the amino acid sequences. Thus, provided that two molecules possess similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules is not found in the other, or if the sequences of amino acid residues are not identical. The term `analog' is meant to refer to a protein that differs structurally from the wild type AHS or AHS transcriptional regulator, but possesses similar activity.

A ``biologically inactive derivative thereof'' is a AHS or a AHS transcriptional regulator that is modified by amino acid deletion, addition, substitution, or truncation, or that has been chemically derivatized, that has reduced function or does not function in the same manner as the wild type protein of SEQ ID NO:15-28. For example, a frame-shift mutation would likely result in reduced function or elimination of function.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

As noted above, the methods of the present invention are suitable for production of any polypeptide of any length, via insertion of the above-described nucleic acid molecules or vectors into a host cell and expression of the nucleotide sequence encoding the polypeptide of interest by the host cell. Introduction of the nucleic acid molecules or vectors into a host cell to produce a transformed host cell can be effected by calcium phosphate transfection,

DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods In Molecular Biology (1986). Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, 1977, *J. Bact.*, 130, 946 and Hsiao *et al.* 1979, *Proc Natl Acad Sci USA* 76, 3829-3833. Once transformed host cells have been obtained, the cells may be cultivated under any physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support host cell growth. Recombinant polypeptide-producing cultivation conditions will vary according to the type of vector used to transform the host cells. For example, certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals or inducing agents to the cell growth medium, to initiate the gene expression resulting in the production of the recombinant polypeptide. Thus, the term "recombinant polypeptide-producing conditions," as used herein, is not meant to be limited to any one set of cultivation conditions. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art.

Following its production in the host cells, the polypeptide of interest may be isolated by several techniques. To liberate the polypeptide of interest from the host cells, the cells are lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high

pressure, or by a combination of the above methods. Other methods of cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the polypeptide may be separated from the cellular debris by any technique suitable for separation of particles in complex mixtures. The polypeptide may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoadsorption, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC), hydroxylapatite chromatography and lectin chromatography.

The recombinant or fusion protein can be used as a diagnostic tool and in a method for producing antibodies against AHS or AHS transcriptional regulator, detectably labeled and unlabeled, or as a bait protein in an assay to isolate proteins or target gene which interact with AHS or AHS transcriptional regulator. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit AHS or AHS transcriptional regulator function, such as host proteins or chemically derived agents or natural or synthetic drugs and other proteins which may interact with the cell to down-regulate or alter the expression of AHS or AHS transcriptional regulator, or its cofactors.

In another embodiment, the present invention relates to monoclonal or polyclonal antibodies specific for the above-described recombinant proteins (or polypeptides). For instance, an antibody can be raised against a peptide

described above, or against a portion thereof of at least 10 amino acids, perferrably, 11-15 amino acids. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the protein (or polypeptide) of the present invention, or a unique portion thereof. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986).

The level of expression of AHS or AHS transcriptional regulator, can be detected at several levels. Using standard methodology well known in the art, assays for the detection and quantitation of AHS or AHS transcriptional regulator RNA can be designed, and include northern hybridization assays, *in situ* hybridization assays, and PCR assays, among others. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985), or Current Protocols in Molecular Biology, Ausubel, F. M. *et al*. (Eds), Wiley & Sons, Inc. for general description of methods for nucleic acid hybridization. Polynucleotide probes for the detection of AHS or AHS transcriptional regulator RNAs can be designed from the sequence. For example, RNA isolated from samples can be coated onto a surface such as a nitrocellulose membrane and prepared for northern hybridization. In the case of *in situ* hybridization of biopsy samples for example, the tissue sample can be prepared for hybridization by standard methods known in the art and hybridized with polynucleotide sequences which specifically recognize AHS or AHS transcriptional regulator RNA. The presence of a hybrid formed between the sample RNA and the polynucleotide can be detected by any method known

in the art such as radiochemistry, or immunochemistry, to name a few.

One of skill in the art may find it desirable to prepare probes that are fairly long and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in the corresponding nucleic acid sequences. In other cases, it may be desirable to use two sets of probes simultaneously, each to a different region of the gene. While the exact length of any probe employed is not critical, typical probe sequences are no greater than 500 nucleotides, even more typically they are no greater than 250 nucleotides; they may be no greater than 100 nucleotides, and also may be no greater than 75 nucleotides in length. Longer probe sequences may be necessary to encompass unique polynucleotide regions with differences sufficient to allow related target sequences to be distinguished. For this reason, probes are preferably from about 10 to about 100 nucleotides in length and more preferably from about 20 to about 50 nucleotides.

The DNA sequence of AHS or AHS transcriptional regulator can be used to design primers for use in the detection of AHS or AHS transcriptional regulator using the polymerase chain reaction (PCR) or reverse transciption PCR (RT-PCR) such as those listed in Table 2 below. The primers can specifically bind to the AHS or AHS transcriptional regulator cDNA produced by reverse transcription of AHS or AHS transcriptional regulator RNA, for the purpose of detecting the presence, absence, or quantifying the amount of AHS or AHS transcriptional regulator RNA by comparison to a standard. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides homologous or complementary to a region of the AHS or AHS transcriptional regulator sequence. Reagents and controls necessary for PCR or RT-PCR reactions are well

known in the art. The amplified products can then be analyzed for the presence or absence of AHS or AHS transcriptional regulator sequences, for example by gel fractionation, by radiochemistry, and immunochemical techniques. This method is advantageous since it requires a small number of cells. Once AHS or AHS transcriptional regulator is detected, a determination whether the cell is overexpressing or underexpressing AHS or AHS transcriptional regulator can be made by comparison to the results obtained from a normal cell using the same method. Decreased AHS or AHS transcriptional regulator may be an indication of reduced virulence of the infecting bacteria, or an indication that tissue-specific or site-specific expression of the gene is reduced.

In another embodiment, the present invention relates to a diagnostic kit for the detection of AHS or AHS transcriptional regulator RNA in cells, said kit comprising a package unit having one or more containers of AHS or AHS transcriptional regulator oligonucleotide primers for detection of AHS or AHS transcriptional regulator by PCR or RT-PCR or AHS or AHS transcriptional regulator polynucleotides for the detection of AHS or AHS transcriptional regulator RNA in cells by *in situ* hybridization or northern analysis, and in some kits including containers of various reagents used for the method desired. The kit may also contain one or more of the following items: polymerization enzymes, buffers, instructions, controls, detection labels. Kits may include containers of reagents mixed together in suitable proportions for performing the methods in accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

In a further embodiment, the present invention provides a method for identifying and quantifying the level of AHS or AHS transcriptional regulator present in a particular sample. Any of a variety of methods which are capable of identifying (or quantifying) the level of AHS or AHS transcriptional regulator in a sample can be used for this purpose.

Diagnostic assays to detect AHS or AHS transcriptional regulator may comprise a biopsy or *in situ* assay of cells from an organ or tissue sections, as well as an aspirate of cells from normal or disease tissue. In addition, assays may be conducted upon cellular extracts from organs, tissues, cells, urine, or serum or blood or any other body fluid or extract. Similarly, the assay may be applied to environmental samples, such as soil, water, and air.

When assaying a sample, the assay will comprise, contacting the sample to be assayed with a AHS or AHS transcriptional regulator ligand or substrate, natural or synthetic, or an antibody, polyclonal or monoclonal, which recognizes AHS or AHS transcriptional regulator, or antiserum capable of detecting AHS or AHS transcripitonal regulator, and detecting the complex formed between AHS or AHS transcriptional regulator present in the sample and the AHS or AHS transcriptional regulator ligand, substrate, or antibody added.

AHS or AHS transcriptional regulator ligands or substrates include for example, a downstream component in the quorum sensing pathway, a substrate for AHS, or an AHS transcriptional regulator interacting protein or DNA binding site, in addition to natural and synthetic classes of ligands and their derivatives which can be derived from natural sources such as animal or plant extracts.

AHS or AHS transcriptional regulator ligands or antibodies, or fragments of ligand and antibodies capable of

detecting AHS or AHS transcriptional regulator may be labeled using any of a variety of labels and methods of labeling for use in diagnosis and prognosis of disease associated with *Burkholderia*. Examples of types of labels which can be used in the present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{57}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{21}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{11}C, ^{19}F, ^{123}I, etc.

Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, ^{46}Fe, etc.

Examples of suitable fluorescent labels include a ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label,etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to ligands and to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of

ordinary skill in the art. Typical techniques are described by Kennedy, J. H., *et al.*,1976 (*Clin. Chim. Acta* 70, 1-31), and Schurs, A. H. W. M., *et al.* 1977 (*Clin. Chim Acta* 81, 1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

The detection of the antibodies (or fragments of antibodies) of the present invention can be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to AHS or AHS response regulator. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

The ligands or antibodies, or fragments of antibodies or ligands discussed above may be used to quantitatively or qualitatively detect the presence of *Burkholderia*. Such detection may be accomplished using any of a variety of immunoassays known to persons of ordinary skill in the art such as radioimmunoassays, immunometic assays, etc. Using standard methodology well known in the art, a diagnostic assay can be constucted by coating on a surface (i.e. a

solid support) for example, a microtitration plate or a membrane (e.g. nitrocelluolose membrane), antibodies specific for either antigen, i.e., AHS or AHS transcriptional regulator, or a portion or either antigen, and contacting it with a sample from a person suspected of having a *Burkholderia* related disease. The presence of a resulting complex formed between the antigen in the sample and antibodies specific therefor can be detected by any of the known detection methods common in the art such as fluorescent antibody spectroscopy or colorimetry. A good description of a radioimmune assay may be found in Laboratory Techniques and Biochemistry in Molecular Biology. by Work, T.S., *et al*. North Holland Publishing Company, N.Y. (1978), incorporated by reference herein. Sandwich assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

The diagnostic methods of this invention are predictive of patients suffering from meliodosis, or glanders disease, or *Burkholderia* related diseases.

The protein can be used to identify inhibitors of AHS activity or AHS transcriptional regulator. Using asssays known in the art for quantitation of AHS, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination of AHS or synthase activity. Knowledge of the mechanism of action of the inhibitor is not necessary as long as a decrease in the activity of synthase is detected. Inhibitors may include agents or drugs which either bind or sequester synthase substrate(s) or cofactor(s), or inhibit the synthase itself, directly, for example by irreversible binding of the agent or drug to the synthase, or indirectly, for example by introducing an agent which binds the synthase substrate. Agents or drugs related

to this invention may result in partial or complete inhibition of synthase activity. Inhibitors of synthase may be used in the treatment or amelioration of glanders disease or meliodosis, and diseases associated with *Burkholderia* infection.

Similarly, agents which reduce the function of AHS transcriptional regulator, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination of response regulator activity. Knowledge of the mechanism of action of the inhibitor is not necessary as long as a decrease in the activity of response regulator is detected. Inhibitors may include agents or drugs which inhibit binding of the signal produced by the synthase to the synthase transcriptional regulator, agents which inhibit binding of the transcriptional regulator itself, directly or indirectly to its target gene, for example by irreversible binding of the agent or drug to the response regulator, by inhibiting multimerization of the enzyme, by blocking the target gene binding site. Agents or drugs related to this invention may result in partial or complete inhibition of trancriptional regulator activity. Inhibitors of AHS transcriptional regulator may be used in the treatment or amelioration of glanders disease or meliodosis, and diseases associated with *Burkholderia* infection.

Agents which decrease AHS or AHS transcriptional regulator RNA include, but are not limited to, one or more ribozymes capable of digesting AHS or AHS transcriptional regulator RNA, or antisense oligonucleotides capable of hybridizing to AHS or AHS transcriptional regulator RNA such that the translation of AHS or AHS transcriptional regulator RNA is inhibited or reduced resulting in a decrease in the level of AHS or AHS transcriptional regulator. These antisense oligonucleotides can be administered as DNA, as

DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Kanoda, Y. *et al.*, 1989, *Science* 243, 375) or as part of a vector which can be expressed in the target cell such that the antisense DNA or RNA is made. Vectors which are expressed in particular cell types are known in the art. Alternatively, the DNA can be injected along with a carrier. A carrier can be a protein such as a cytokine, for example interleukin 2, or polylysine-glycoprotein carrier. Such carrier proteins and vectors and methods of using same are known in the art. In addition, the DNA could be coated onto tiny gold beads and said beads introduced into the skin with, for example, a gene gun (Ulmer, J. B. *et al.*, 1993, *Science* 259, 1745).

Alternatively, antibodies, or compounds capable of reducing or inhibiting the synthase or the synthase transcriptional regulator, that is reducing or inhibiting either the expression, production or activity of these proteins, such as antagonists, can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the synthase-reducing or inhibiting agent is produced. In addition, co-factors such as various ions, i.e. $Ca2^{+}$ or factors which affect the stability of the enzyme can be administered to modulate the expression and function of synthase. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, synthase-inhibiting compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of infection or implanted so that the synthase-

inhibiting compound is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.*(1991) *J. Neurosurg.* 74, 441-446. These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of synthase. Similarly, agents which are capable of negatively affecting the expression, production, stability or function of synthase, are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of synthase. An amount is said to be sufficient to ``effect'' the inhibition or induction of synthase if the dosage, route of administration, etc. of the agent are sufficient to influence such a response.

In providing a subject, specifically equine or human, with agents which modulate the expression or function of synthase to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

A composition is said to be ``pharmacologically acceptable'' if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a ``therapeutically effective amount'' if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in

admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences [16th ed., Osol, A. ed., Mack Easton PA. (1980)]. In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)-microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in

macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The present invention also provides kits for use in the diagnostic or therapeutic methods described above. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the compositions of the invention.

The kits of the invention may comprise one or more of the following components, one or more compounds or compositions of the invention, and one or more excipient, diluent, or adjuvant.

In another embodiment, the present invention describes *Burkholderia* strains, specifically *B.mallei* and *B. pseudomallei* strains, merodiploid strains and other mutants in various AHS and LuxR genes (Table 1). Any alteration of one or more of AHS or AHS transcriptional regulator gene which results in an avirulent, live attenuated strain is part of the present invention. The strain *GB8::bpmI3* is avirulent, but still able to produce a capsule similar to wild type. All animals (40%) challenged with wild type *B. mallei* after aerosol exposure to *GB8::bpmI3* survived and no recoverable mutants were isolated from spleen extracts. *GB8::bpmI1* also showed a significant reduction in virulence however, exposures to *GB8::bpmI1* prior to challenge resulted in 0% survival of the experimental group. Viable organisms were recovered from the spleens of animals exposed to *GB8::bpmI1*. Other mutant strains wherein more that one synthase is missing, or a combination of synthase genes and response regulator genes are altered are likely to produce a mutant strain with the desired virulence and ability to protect against challenge. Specifically, by deleting the

bmaI1, bmaI3, and bmaR5, genes, a combinatorial effect in the reduction of virulence observed for a single mutant should be beneficial in a single strain. Preferably, the mutation introduced is designed to be non-revertable, i.e. will not revert to wild-type.

The mutant strains, e.g. *GB8::bpmI1* strain, or non-revertant mutant strains of *B. mallei* or *B. pseudomallei*, may function as a gene or gene product delivery system since the strain has reduced virulence, can penetrate the tissue, resides in the tissue for a specified period of time, and is eventually cleared from the tissue by the host. For example, it is envisioned that an antigen of interest could be delivered to an organ, specifically the lungs, which is naturally invaded by the bacterial delivery agent in a patient where the antigen can provide benefit. The antigen can be introduced into the bacterial delivery agent in a second plasmid. Alternatively, a second plasmid could be used to provide a source of vaccine antigen for pathogens found in organs naturally invaded by *Burkholderia* such as a systemic invasion, spleen, or kidney, lung, central nervous system, eye, to name a few.

Such strains represents a safe delivery vehicle and are advantageous because they can carry one or more compounds and can be genetically engineered to carry one or more nucleic acid molecules capable of effecting gene therapy and/or of encoding one or more proteins and/or RNA molecules. The compound of interest can be carried by such a strain, e.g. *GB8::bpmI1* within the bacteria cell, on the membrane surface, in the capsule, spanning the membrane, withing the periplasm, and combinations thereof. At least some of the compound of interest remains associated with the bacteria at least until the bacteria reaches its target, or site of action (e.g. the bloodstream, interstitial tissue,

or a cell), at which point it is also possible that a compound carried by the bacteria may be released. As used herein, a compound capable of protecting an animal or plant from disease is a compound that when administered to an animal or plant can prevent a disease from occuring and/or cure or alleviate disease symptoms or cause. Examples of diseases from which to protect an animal or plant include, but are not limited to, infections, genetic defects and other metabolic disorders. Such classes of diseases can lead to abnormal cell growth (e.g., benign or malignant neoplasia, hyperplastic syndromes), degenerative processes, and/or immunological defects as well as to a number of other disorders.

In accordance with the present invention, compounds included in the above-described delivery vehicles can have a variety of functions. Delivery vehicles of the present invention preferably include compounds capable of stimulating an immune response, compounds capable of suppressing an immune respone, toxic compounds, compounds capable of inhibiting transcription of a gene, compounds capable of inhibiting translation of a gene, compounds capable of inhibiting the ability of an infectious agent to produce progeny, compounds capable of replacing a defective gene, compounds capable of replacing a defective protein (including nucleic acid molecules capable of encoding such proteins and mimetopes of such proteins) and/or biological response modifiers (e.g., cytokines, such as lymphokines and monokines, as well as other growth modulating factors), and mixtures thereof. Examples of such compounds include, but are not limited to, antibiotics, antibodies, antifungal compounds, antigens, antiparasite compounds, antisense compounds, antiviral compounds, chemotherapeutic agents, cytokines, growth modulating factors (including both growth stimulants and suppressants), herbicides, hormones,

immunosuppressants, nucleic acid-based drugs (e.g., DNA- or RNA-based drugs), nucleic acid molecules comprising coding regions, nucleic acid molecules comprising regulatory sequences, nucleoside analogs, other oligonucleotides, peptide analogs, peptides, pesticides, prodrugs (e.g., compounds that are activated at the site of action), other proteins, ribozymes, steroids, toxins, and/or vitamins.

Cell types naturally targeted by *Burkholderia* include, but are not limited to, lung, spleen, and kidney, among others.

The present invention includes the delivery of a composition comprising the delivery vehicle of the present invention to an animal or to a cell in culture. Such compositions can be delivered to an animal either *in vivo* or *ex vivo*, or can be delivered to cells *in vitro*. Such administration can be systemic, mucosal, and/or proximal to the location of the targeted cell type. Examples of routes to administer bacteria *in vivo* include aural, bronchial, genital, inhalatory, nasal, ocular, oral, parenteral, rectal, topical, transdermal, and urethral routes.

Ex vivo delivery refers to a method that includes the steps of contacting a population of cells removed from an animal with a composition comprising the delivery vehicle of the present invention under conditions such that the bacteria is adsorbed by targeted cell types and returning the contacted cells to the animal. Such a delivery method is particularly useful in the treatment of cells involved in hematopoiesis and the immune response as well as in the treatment of tumors.

In vitro delivery refers to the delivery of the delivery vehicle of the present invention to a population of cells (which can also include tissues or organs) in culture.

Methods to prepare and administer compositions via these routes are well known to those skilled in the art. A preferred single dose of a bacteria vehicle of the present invention is from about 1 x 10^5 to about 5 x 10^7 bacterial cell equivalents per kilogram body weight of the organism being administered the composition.

The mutant strains described above can be used for vaccine. In particular, the vaccine strain of the invention having a non-revertant mutation in *bmaI3* and/or *bmaI1* for a *B.mallei* vaccine to protect against glanders disease, or *bpmI3* or *bpmI1* for *B.pseudomallei* vaccine to protect against meliodosis. The similarity of the *B.mallei* and the *B.pseudomallei* genomes and diseases indicates that one vaccine should work against both diseases. The vaccine strain can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized compositions will typically be maintained at about 4°C. When ready for use the lyophilized composition is reconstituted in a stabilizing solution, e.g., saline or comprising Mg^{++} and HEPES, with or without adjuvant, as further described below.

Thus the vaccine of the invention contains as an active ingredient an immunogenically effective amount of a non-revertant, avirulent, *B. mallei or B.pseudomallei* strain having a mutation in one or more AHS gene or a mutation in one or more transcriptional regulator gene as described herein. The vaccine strain may be introduced into a host, particularly humans or equine, with a physiologically acceptable carrier and/or adjuvant or with another mutant strain having a different mutation in the same or different AHS gene or AHS transcriptional regulator gene to increase the effectiveness and/or safety of the vaccine. Useful carriers are well known in the art, and include, e.g.,

water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like.

Administration of the vaccine strain disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the bacteria (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the bacteria to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally), by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the bacteria as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. As a result of the vaccination the host becomes at least partially or completely immune to *B.mallei* infection, or resistant to developing moderate or severe *B. mallei* infection.

The vaccine composition containing the vaccine strain of the invention can be administered to a person susceptible to or otherwise at risk of *Burkholderia* infection to enhance the individual's own immune response capabilities. Such an amount is defined to be a ``immunogenically effective dose''. In this use, the precise amount again depends on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1×10^5 to about 5×10^7 bacteria cell equivalents per kilogram body weight of the organism being administered the composition. In any event, the vaccine formulations should provide a quantity of the vaccine strain of the invention sufficient to effetively protect the patient against serious or life-threatening *Burkholderia* infection.

In some instances it may be desirable to combine the *Burkholderia* vaccines of the invention with vaccines which induce protective responses to other agents.

Single or multiple administration of the vaccine compositions of the invention can be carried out. Multiple administration may be required to elicit sufficient levels of immunity. Levels of induced immunity can be monitored by measuring amount of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and

6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors and thought to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

The following MATERIALS AND METHODS were used in the examples that follow.

Bacterial strains and plasmids:

The bacterial strains and cloning vectors in this study are described in Table 1. *B. thailandensis, Chromobacterium violaceum CV026, Agrobacterium tumefaciens (A136), Escherichia coli* and *B. pseudomallei* were cultured in Luria-Bertani (LB) broth or on LB agar at 30°C or 37°C as required. *B. mallei* was cultured in LB broth or LB agar with the addition of 4% glycerol. For the screening of recombinant clones *E. coli* was grown on LB plates containing 100 ug/ml ampicillin or 25 ug/ml kanamycin, 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG), and 50 ug/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-Gal).

Cloning the *B. pseudomallei* quorum sensing genes.

Genomic DNA from *B. pseudomallei* NCTC 4845 was digested with ClaI and ligated into similarly digested pBluescript KS+. The plasmids were transformed into *E. coli* JM109 pSB401 by electroporation. Plasmid pSB401 contains all the genes from *Photobacterium fischeri* required for bioluminescence except *luxI*. Therefore, only inserts in pBluescript encoding a *luxI* homolog capable of producing AHLs was able to induce bioluminescence, and thus produce colonies emitting light.

AHL reporter assays.

DW503 quorum mutants were analyzed for AHL synthesis using bioreporter strains that respond to exogenously secreted AHLs or varying composition. Using a cross feeding assay, the reporter strain (CVO26 or A136) was streaked vertically on a 100 X 15 mm LB Petri plate, while the mutant strain was inoculated horizontally. For analysis, incorporation A136, strains were streaked onto LB plates containing 50 ug/ml X-Gal and incubated for 24-48 hrs at 37°C. For CVO26, plates were generally incubated for 24 hr at 30°C (9+10). Pigment production by CVO26 or bluing of A136 at the junction site indicates AHL synthesis and secretion.

Exoproduct secretion and motility analysis.

Siderphore activity was measured on CAS agar plates using methods previously described. Briefly, DW503 mutants and wild type DW503 were tooth picked onto CAS plates and incubated for 24-48 hrs at 37°C. Iron removal, indicative of siderphore secretion, was assayed by measuring the blue-orange halo surrounding the inoculation site. Protease and lipase secretion was monitored using methods described by DeShazer et al. To assay for hemolysis and or rhamnolipid

biosynthesis, colonies were tooth picked onto 5% sheep blood agar plates and incubated for 24-72 hrs at 37°C. Hemolysis was indicated by a clearing of the erythrocytes around the site of inoculation. Twitching and swarming motility was examined using methods described by Kohler et al. and Reimmann et al. Plates were incubated at 30°C for 48-72 hrs.

AHL extraction, TLC, and MS analysis.

Extraction of AHLs from culture supernatants and preparative TLC was performed as described by Shaw et al. TLC scrapings tentatively identified as containing AHLs were extracted three times with 1 ml of methylene chloride (HPLC grade; B&J, VWR Scientific, Bridgeport, NJ). Stationary phase material was pelleted by centrifugation at 4,000 rpm for 10 min. Supernatants were pooled and evaporated to dryness at 50°C under a gentle stream of nitrogen. Dried samples were reconstituted in 100 ul of 50% acetonitrite (HPLC grade) in 0.1% formic acid.

Aliquots (20 ul) were injected onto a PepMap C18 column (150 X 1mm, 5 u, 100A) (LC Packings, San Francisco, CA). An ABI 140B syringe pump (Applied Biosystems, Foster City, CA) provided a flow rate of 50 ul/min, which was used with a 20 min gradient of 0 to 100 % B to elute the compounds of interest. Solvent A consisted of 0.1% formic acid, and solvent B contained 0.1% formic acid in 95% acetonitrile. The column effluent was directed into a Finnigan DECA ion trap mass spectrometer fitted with an API II electrospray interface. The transfer capillary temperature was 350°C. Full scan, positive ion spectra were acquired by scanning from m/z 100 to m/z 335 in 1.5 sec. For identification, components were fragmented by collision-induced dissociation of the respective [M+H]+ ion using a relative collision energy setting of 19. These spectra were acquired by

scanning from m/z 50 to m/z 335 in 1.5 sec. MS/MS spectra of unknowns were compared to those of standard compounds acquired under the same instrumental conditions for confirmation of identity.

Primer design

Primer design for each allele was based upon reference to the *B. pseudomallei* K96243 genome project (http://www.sanger.ac.uk/). Genomic DNA for PCR amplification was purified using the MasterPure™ DNA purification kit according to the manufacturer's instructions (Epicentre Technologies, Madison, WI). Internal gene fragments were PCR amplified with the primer pairs listed in Table 2 using the following conditions: one cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, followed by a final 7 min extension at 72°C. For confirming site-specific integration, the extension time was increased to 4 min. All PCR reactions were performed with the Epicentre FailSafe kit using buffer ``J'' (Epicentre Technologies). Reactions were analyzed on a 0.8% agarose gel containing ethidium bromide (43) and subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Ligations were transformed into One Shot® chemically competent *E.coli* (Invitrogen) and screened by standard methods (43).

Mutant construction and confirmation:

Disruption cassettes were made by digesting pCR2.1-TOPO containing internal gene amplicons for each of the eight quorum loci with *EcoR*1 (New England Biolabs, Beverly, MA) for 1 hr at 37°C. Digestions were heat inactivated and subcloned into the suicide vector pGSV3 (McClean et al., 1997, supra) using the Epicentre Fast-Link DNA ligation kit (Epicentre Technologies). Ligations were chemically

transformed as described above and screened on LB plates containing 10 μg/ml of gentamycin (Sigma). Random colonies (five from each transformation) were inoculated into 2 ml of LB broth containing 10 μg/ml of gentamycin and incubated at 37°C for 16-18 hr with agitation. Plasmid DNA was purified using the Wizard Plus Miniprep kit (Promega, Madison, WI), digested as described above, and analyzed on a 0.8% agarose gel with ethidium bromide. Clones containing inserts were electrically transformed into *E. coli* SM10 and mobilized into *B. thailandensis* DW503, Ricky, is this supposed to be B. mallei, or B. pseudomallei(Simon et al., 1989, supra). Transconjugants were selected on LB plates containing 10 μg/ml of gentamycin and 15 μg/ml of polymyxin (Sigma). Genomic DNA from transconjugants, three mutants from each mating experiment, was purified using methods described above. Site-specific integration, indicated by a 3.0 Kb increase in amplicon size corresponding to the suicide vector, was confirmed using PCR methods previously described for target gene amplification incorporating an extension time of 4 min.

Whole body Aersol exposures:

Approximately 48 hr prior to challenge 3 ml cultures were individually inoculated with wild-type *B. mallei* and each quorum mutant and incubated for 24 hr at 37°C. A 1 ml aliquot from the 3 ml overnight cultures was used to inoculate 25 ml of LBG. Cultures were incubated at 37°C for 18 hr, optical densities (OD_{660}) measured, and 10 ml (approximately 10^9 colony forming units/ml) was delivered to groups of 10 mice via nebulization using methods described by Jeddeloh et. al.(2002, supra). Chamber concentration was determined by CFU enumeration from air samples collected within the exposure compartment and the relative inhaled

dose was deciphered by factoring the number of respirations for 6 week-old BALB/c mice (Jeddeloh et al., 2002, supra).

Organ loads:

The relative bacterial loads within the spleen, liver, and lungs of female BALB/c mice challenged with each *B. mallei* and *B. pseudomallei* mutant and wild type strains was assayed over a 5 day period. Animals were humanly euthanized using CO_2, organs extracted, and homongized in 1 ml of sterile PBS. Organ extracts were serially diluted, plated onto LBG containing 10 μg/ml of gentamycin, and incubated for 48 hr at 37°C.

Example 1

Using the *cepIR* and *lasIR* genes as digital probes, several AHSs and transcriptional regulators were identified within the K96243 genome. Given the genetic similarity between *B. thailandensis* DW503 and *B. pseudomallei* 1026b, only small internal gene amplicons corresponding to each quorum allele were PCR amplified (Figure 1) and sequenced from each strain. Nucleotide comparisons between the *B. thailandensis* DW503 and *B. pseudomallei* 1026b quorum genes demonstrated significant DNA homology (data not shown). PCR amplification and BLASTX search results further confirmed that the *B. thailandensis* DW503 genome encodes three AHS and five putative transcriptional regulators belonging to the LuxIR family of quorum proteins.

Example 2

Gene alignments for *B. pseudomallei.*

The identified *B.pseudomallei/mallei* quorum genes share similarity with AHS and AHL receptors from *Burkholderia vietnamiensis, Ralstonia solanaserum, Burkholderia multivorans,* and *P. aeroginosa.* All of the AHS genes identified encode AHL-synthases of the autoinduce 1 (AI 1) sub-family and therefore are conceivably involved with intraspecies communication. Neither the B. mallei nor B. pseudomallei genome contained a *V. fishcheri lusX*-like AI 2 subfamily synthase. Merodiploids in each of the eight genes identified were constructed and phenotypically characterized using multiple assays. Disruption of *bpmI1 and bpmRI* affected quorum signaling in the *Chromobactirium violaceum* reporter strain CV026. In contrast, disruption of the *bpmI3, R3* and *R5* ORFs induced a hyper-hemolytic phenotype and enhanced siderophore secretion in *B. thailandensis.* In addition, lipase secretion, swarming and twitching motility were also effected by quorum disruptions. Loss-of-function mutations that produce gain-of-function phenotypes indicate this quorum network operates using both positive and negative signaling.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Reference or Source
Escherichia Coli		
SM10	Mobilizing strain; RP4 *tra* genes; Km^r	Simon etal,1983
TOP10	Used for cloning and blue-white screening	Invitrogen
Burkholderia Mallei		
ATCC 23344	Human isolate	USAMRIID
Burkholderia pseudomallei		
DD503	Δ(*amrR-oprA*) *rpsL* (Sm^r) AG^s Tc^s	Moore et al., 1999
BTRJ10	DD503 derivative; I1::pGSV3; Gm^r	This study
BTRJ12	DD503 derivative; I2::pGSV3; Gm^r	This study
BTRJ13	DD503 derivative; I3::pGSV3; Gm^r	This study
BTRJ14	DD503 derivative; R1::pGSV3; Gm^r	This study
BTRJ15	DD503 derivative; R2::pGSV3; Gm^r	This study
BTRJ16	DD503 derivative; R3::pGSV3; Gm^r	This study
BTRJ17	DD503 derivative; R4::pGSV3; Gm^r	This study
BTRJ18	DD503 derivative; R5::pGSV3; Gm^r	This study
BTRJ19	ATCC 23344 derivative; I1::pGSV3; Gm^r	This study
BTRJ20	ATCC 23344 derivative; I3::pGSV3; Gm^r	This study
BTRJ21	ATCC 23344 derivative; R1::pGSV3; Gm^r	This study
BTRJ22	ATCC 23344 derivative; R3::pGSV3; Gm^r	This study
BTRJ23	ATCC 23344 derivative; R4::pGSV3; Gm^r	This study
BTRJ24	ATCC 23344 derivative; R5::pGSV3; Gm^r	This study
Plasmids		
pGSV3	Mobilizable suicide vector; Gm^r	McClean et al. 1997
pCR2.1-TOPO	TA cloning vector; Km^r Ap^r	Invitrogen
pBHR1	Mobilizable broad-host-range vector; Km^r Cm	MoBiTec
pRUI1	Contains a 369 bp PCR product from the 1026b I1 synthase gene	This study
pRUI2	Contains a 360 bp PCR product from the 1026b I2 synthase gene	This study
pRUI3	Contains a 398 bp PCR product from the 1026b I3 synthase gene	This study
pRUR1	Contains a 397 bp PCR product from the 1026b R1 transcriptional regulator	This study
pRUR2	Contains a 424 bp PCR product from the 1026b R2 transcriptional regulator	This study
pRUR3	Contains a 402 bp PCR product from the 1026b R3 transcriptional regulator	This study
pRUR4	Contains a 391 bp PCR product from the 1026b R4 transcriptional regulator	This study
pRUR5	Contains a 401 bp PCR product from the 1026b R5 transcriptional regulator	This study

TABLE 2. Primers used for PCR amplification of internal gene amplicons

Gene[a]	Primer sequence	Amplicon size
bthI1	F 5'-CCGCGACGACGACGGGGAAATC-3', SEQ ID NO:29 R 5'-TCGATCCAGCACGCGACGACCAT-3', SEQ ID NO:30	369 bp
bthI2	F 5'-ATAAGCGCCGCGCAACTGGATTCC-3', SEQ ID NO:31 R 5'-CAGGATCGCCGTATTGCGGTGAGC-3', SEQ ID NO:32	360 bp
bthI3	F 5'-TCGCGGGCCGATTGAACGAACTGC-3', SEQ ID NO:33 R 5'-GAGCGACGCGGCCACCGTGAGCAC-3', SEQ ID NO:34	398 bp
bthR1	F 5'-CGGCTTCGAATATTGCTGCTATGG-3', SEQ ID NO:35 R 5'-GAGAAAACGGCTCATCAGCGAGTG-3', SEQ ID NO:36	397 bp
bthR2	F 5'-AGCGACCGGCCCGTGACCTGGAG-3', SEQ ID NO:37 R 5'-CGGCCTGTATCTTGTTCGTGGAG-3', SEQ ID NO:38	424 bp
bthR3	F 5'-AGACGTCGTCTCGCTGCACTATCC-3', SEQ ID NO:39 R 5'-ACCCACGTGAGGCACATCTGTTCG-3', SEQ ID NO:40	402 bp
bthR4	F 5'-GGCGTTCGACAGATGAAACACGAC-3', SEQ ID NO:41 R 5'-GCTCATCTGGCACGACGACCTCTA-3', SEQ ID NO:42	391 bp
bthR5	F 5'-CGCGTGCCGTGGCCGCTGTCCA-3', SEQ ID NO:43 R 5'-CCGCGCTCCGGGTCCGCCATCAG-3', SEQ ID NO:44	401 bp

[a] *bthI1-I3* correspond to AHSs while *bthR1-R5* represent transcriptional regulators.

The *B. pseudomallei* quorum loci are similar to those of *B. mallei*. The loci are structurally complex and are flanked by several characterized and unknown proteins. The *bpmIR1* and *bpmIR2* alleles are divergently transcribed while the *bpmR4* and *bpmR5* are in a gene cluster that contains no putative AHL synthase. Intergenic disruption of this type have been identified in several species of Gram negative bacteria (McClean et al., 1997, supra; Moore et al., 1999, supra). Numerous orf's adjacent the *bpmIR* genes were identified in this study that have not been shown to be quorum regulated. Lewenza *et al.* (1998, supra) reported a Mg^{2+} transport protein located downstream from *cepR*. The *bpmR1*, most similiar to *bviR*, also contained a Mg^{2+}

transport protein located downstream. The *bpmIR2* loci are separated by a 3 kb intergenic region that contains two GeneMark predicted proteins with no similarity to known enzymes and a putative ion transport protein. Conway and Greenberg (2002, supra) reported that *B. vietnamiensis* produces an antibiotic that is potentially regulated by quorum sensing. Interestingly, positioned down stream from the *bpmI2* gene is an orf that contains homology to several proteins involved antibiotic synthesis. Also, located upstream from the *bpmR3* is a putative long-chain fatty-acid-CoA ligase protein. Conway and Greenberg (2002, supra) also reported the presence of a *fabF*-like gene located downstream from *bviR*. Mutational analysis of this gene indicated that *fabF* was not involved in acyl-ACP generation for *bviI* and did not influence AHL synthesis in *B. vietnamiensis*. The remaining *bpm* genes are flanked by several orf's with little or no similiarity to known gene products. None of the quorum genes characterized in this study are structurally orientented in the tail-tail position as seen in *P. aeruginosa*. The *bpmIR1* and *bpmIR2* are all divergently transcribed and contain intergenic regions while the *bpmR4* and *bpmR5* are orphaned for a corresponding AHS. Both of these genes exhibited similarity to LuxR type proteins and disruptions in these alleles resulted in verifiable phenotypes.

Example 2

B. thailandensis quorum sensing mutants.

To assay for hemolysis and/or rhamnolipid biosynthesis, colonies were tooth picked onto 5% sheep blood agar plates and incubated at 37 C for 24-72 hours. Hemolysis was indicated by a clearing of the erythrocytes around the site

of inoculation. Analysis of *B. thailandensis* and the engineered quorum mutants revealed that mutations in *bpm::R1, bpm::R2,* and *bpm::R4* produced zones of hemolysis equivalent to that of wild type DW503. In contrast, mutations in *bpm::I1* exhibited slight hemolysis while *bpm::I2, bpm::I3, bpm::R3, and bpm::R5* disruptions revealed hyperhemolytic phenotypes with extensive beta hemolysis.

Twitching and swarming motility were examined using methods described by Reimmann et al. Plates were incubated at 30 C for 48-72 hrs. Mutations in the *bpm::I2, bpm::R1,* and *bpm::R3* loci appeared to induce a defective twitching phenotype. Wild type DW503 colonies display a saucoidal symmetrical morphology without visible pigmentation. Mutations in the *bpm::I3, bpmIIR3* exhibited a wrinkling phenotype in which the cells proliferated from the center of the inoculation site and grew on the surface of the underlying colony. Interestingly, *bpm::R3* produced a faint orange pigment and displayed extensive wrinkling without the glistening appearance of DW503. Like twitching motility, quorum sensing also played a regulatory role for swarming motility in *B.thailandensis*. On swarm plates, DW503 grew in a irregular and spreading fashion at 24 hrs and completely colonized the entire plate after 36 hrs.

All the *B.thailandensis* quorum mutants produced this glistening exopolysaccharide on swarm plates. Mutations in *bpm::I2 and bpm::R5* exhibited a defective swarming motility phenotype indicated by the inability to colonize 0.5% agar plates. In contrast, disruption of the *bpm::R1* locus resulted in an enhanced capability of plate colonization.

Siderphore activity was measured on CAS agar plates using methods previously described. Briefly, DW503 mutants

and wild-type DW503 were tooth picked onto CAS plates and incubated for 24-48 hrs at 37 C. Iron removal, indicative of siderphore secretion, was assayed by measuring the blue-orange halo surrounding the inoculation site. Protease and lipase secretion was monitored using methods described by DeShazer et al. Using each of the *B.thailandensis* AHL synthase and transcriptional regulator mutants, plate assays for hemolysis and detection of protease, siderphore, lipase and phospholipase C (PLC) were analyzed. Both PLC (egg yold plates) and protease production (3% skim milk) were not altered by any of the *B. thailandensis* quorum mutants tested in this study. Unlike protease synthesis, lipase secretion is both positively and negatively regulated by the *B.thailandensis* quorum sensing network. Mutations in the *bpm::I2* and *bpm::R2* genes produced a reduction (26.8% and 39%) in lipase biosynthesis while mutations in *bpm::I1* and *bpm::R1* (46.3% and 80.4%), *bpm::I3* and *bpm::R3* (70.2% and 107%), *bpm::R4* (58.5%), and *bpm::R5* (46.3%) demonstrated elevated levels of lipase secretion in comparison to DW503. Siderphore production was slightly enhanced in *bpm::I3* and moderately elevated in *bpm::R3* mutants. Levels of siderphore secretion for *bpm::R1*, *bpm::R2*, *bpm::R4*, and *bpm::R5* were equivalent to that of DW503.

Example 3

Site-specific integration of the internal gene fragment with the target *B. mallei* gene was confirmed using PCR with whole gene primers. Following recovery and confirmation, the mutants were subjected to a series of in vitro tests to determine which AHL signaling molecules they synthesize. The results of this analysis suggest that the BmaI1 and BpmI1 direct the synthesis of C_8-HSL and the *bmaI3* and *bpmI3*

genes encode proteins that produce C_6-HSL. In contrast, the *B. pseudomallei* BpmI2 allows for the biosynthesis of *N*-decanoyl homoserine lactone. A thin liquid chromatography (TLC) based reporter assay (McClean, K. H. et al., 1997, Microbiology 143, 3703-3711; Zhang,Z. and L. S. Pierson III, 2001, Appl. Environ. Microbiol. 67, 4305-4315) in conjunction with mass spectrometry was used to confirm these results.

The AHS merodiploids in *B. mallei* were evaluated in whole body aerosol models (Jeddeloh, J. et al., 2003, Infect. Immun. 71, 584-587).

Female BALB/c mice were sprayed with approximately 50 LD50 (10,000CFU) using methods developed by USAMRIID. Challenges were performed by the aerobiology division within USAMRIID in a BL3 containment suite. Mice were sacrificed at day 7 and spleens were extracted. After homogenizing, 100 ul of a 5 ml extract was plated onto LB containing 4% glycerol (LBG) with 10 ug/ml gentamycin. To enumerate wild type *B. mallei*, extracts were plated onto LBG and incubated for 24-36 hrs at 37 C.

Both the GB8::*bpmI1* and GB8::*bpmI3* mutants were avirulent. Interestingly, the GB8::*bpmI1* mutans were still able to colonize the spleen, liver and lungs of infected animals at days 1-5 post exposure (Figure 3 and Table 3). At day 30, only the spleens contained recoverable GB8::*bpmI1* mutants (Figure 3). In contrast, the *GB8::bpmI3* AHS mutants initially colonized the spleen and liver (Figure 3) at days 1-5 but were cleared at day 30 (data not shown). Unlike the spleen and liver, the lungs of infected animals receiving GB8::*bpmI1* and GB8::*bpmI3* mutants were sterile by day 4 (Figure 3) (The mixture of *bmaI1* and *bmaI3* mutants were not able to complement each other in trans.) Of the

transcriptional regulator mutants, disruption of the *bmaR3* and *bmaR5* genes had the greatest effect on virulence. As with the *B. mallei* AHS mutants, the lungs of animals infected with LuxR mutants were cleared by day 4 (Figure 3) post exposure. The spleen and liver of animals challenged with the *B. mallei* transcriptional mutants contained low bacterial loads in comparison to wild-type *B. mallei* (Figure 3).

Table 3-Spleen loads from *B.mallei* aerosol challenges

Organism Sprayed	Inhaled dose	Gmr spleen isolates	Gms spleen isolates	Total Recovered	Percent mutants	Percent wild-type
GB8::bpmI1	10906	3	17	19	10.5	89.5
GB8::bpmI3	9800	0	0	0	0	0
GB8::bpmR1+ WT GB8	10345	13	57	70	23	77
GB8::bpmR3+ WT GB8	9975	17	54	71	31	69
GB8::bpmR5+ WT GB8	11675	3	99	102	9	91
GB8::DD3008	9468	0	41	41	0	100
GB8::bpmI1+ GB8::bpmI3	8200	3	1	14	92.4	7.6

[a]A total of 10 mice were sprayed for each group and spleens were processed as described. GB8 is a Great Britain isolate and was used to create the merodiploids in this study. DD3008 is a *B. mallei* capsule mutant that fails to cause mortality in mice aerosol exposures. WT depicts wild type *B. mallei*.

[b]The inhaled dose was calculated by plating dilutions of nebulizer samples taken from each exposure pan containing 10 mice. The mathematical model for calculating the inhaled CFU's was developed by the aerobiology division at USAMRIID.

Bacterial loads were numerated in triplicate by sacrificing 3 mice from each exposure group.

Unfortunately, the reduction in virulence observed in *B. mallei* was not as profound in *B. pseudomallei*. Of the eight *B. pseudomallei* quorum sensing mutants generated, only the DD503::*bpmI3* displayed a reduction in pathogenicity using an aerosol BALB/c model (Figure 5). Only 30% of the DD503::*bpmI3* experimental group was lost over the 30 day experimental window in contrast to 100% for wild type DD503.

To date the only definitive virulence factor associated with the pathogenicity of *B. mallei* is extracellular capsule (DeShazer, D. et al., 2001, supra). All of the *B. mallei* quorum sensing mutants tested in this study produce capsule even those with reduced virulence. This is of significant importance indicating that this study has identified novel and previously unknown regulators of virulence and virulence gene expression.

Animals receiving the GB8::*bpmI1* and GB8::*bpmI3* mutants survived their initial aerosol challenge and were exposed again (Figure 4) 21 days post exposure. Approximately 3 weeks following this secondary boost, animals were challenged with wild-type *B. mallei* ATCC 23344 (or GB8) by whole body aerosolization with 10 LD50s (around 10,000 CFU). The animals exposed to mutant derivatives received a similar dose for the initial and secondary challenges.
Surprisingly, over a 21 day period, approximately 40% of the vaccinated animals exposed to *GB8::bpmI3* survived while all memebers in the un-vaccinated group perished within 3 days. To our knowledge, the best performing whole-cell vaccine preparation only yields an extension in time to death by 1-2 days. Protection to 21 days has not been observed for a glanders vaccine previously.